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Evolution of Developmental Control Mechanisms

Diverging functions of Scr between embryonic and post-embryonic development in a hemimetabolous insect, *Oncopeltus fasciatus*

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ABSTRACT

Hemimetabolous insects undergo an ancestral mode of development in which embryos hatch into first nymphs that resemble miniature adults. While recent studies have shown that homeotic (hox) genes establish segmental identity of first nymphs during embryogenesis, no information exists on the function of these genes during post-embryogenesis. To determine whether and to what degree hox genes influence the formation of adult morphologies, we performed a functional analysis of *Sex combs reduced* (*Scr*) during post-embryonic development in *Oncopeltus fasciatus*. The main effect was observed in prothorax of *Scr*-RNAi adults, and ranged from significant alterations in its size and shape to a near complete transformation of its posterior half toward a T2-like identity. Furthermore, while the consecutive application of *Scr*-RNAi at both of the final two post-embryonic stages (fourth and fifth) did result in formation of ectopic wings on T1, the individual applications at each of these stages did not. These experiments provide two new insights into evolution of wings. First, the role of *Scr* in wing repression appears to be conserved in both holo- and hemimetabolous insects. Second, the prolonged *Scr*-depletion (spanning at least two nymphal stages) is both necessary and sufficient to restart wing program. At the same time, other structures that were previously established during embryogenesis are either unaffected (T1 legs) or display only minor changes (labium) in adults. These observations reveal a temporal and spatial divergence of *Scr* roles during embryonic (main effect in labium) and post-embryonic (main effect in prothorax) development.

Introduction

A large portion of our current knowledge of the evolution of new morphologies has been inferred from studies of insects, particularly their appendages (Angelini and Kaufman, 2004, 2005a,c; Carroll, 1995; Carroll et al., 1995, 2001; Hughes and Kaufman, 2000; Mahfooz et al., 2004, 2007; Randsholt and Santamaria, 2008; Weatherbee et al., 1999; Wilkins, 2002). The evolution of wings and legs was instrumental in the radiation and diversification of insects and some of the best-documented examples of regulatory evolution come from investigations of the molecular basis of modifications in these structures (Brunetti et al., 2001; Carroll et al., 1995; Gompel et al., 2005; Monteiro, 2008; Weatherbee et al., 1999). To a large degree, the differences in appendage morphology can be explained by alterations in function, regulation, and expression of common body and appendage patterning genes (Angelini and Kaufman, 2004, 2005c; Angelini et al., 2005; Carroll, 1995; Hughes and Kaufman, 2002; Mahfooz et al., 2007; Rogers et al., 1997; Ronshaugen et al., 2002). Some of these common developmental regulators, such as hox genes, also control the identity of body segments and their pigmentation (Hughes and Kaufman, 2002; Jeong et al., 2006; Lohmann et al., 2002). However, the molecular mechanisms governing the structural diversity of segments (i.e. size and shape) *per se* have remained largely unexplored. Similar to appendages, thoracic segments themselves also exhibit an extraordinary array of differences with regard to their size, shape, pigmentation and function. The largest diversification is observed in the prothorax (T1), which in some insects is drastically reduced (Diptera, flies), while in others it can be quite enlarged, concealing the head (Blattaria, cockroaches). The extent of variation in T1 morphologies is most prominent in hemimetabolous insects, even becoming a hallmark lineage-specific trait in various true bugs (Hemiptera). In families such as Membracidae (treehoppers) the pronotum may extend the entire length of the body and take on myriad elaborate shapes and modifications. At present, the molecular mechanisms responsible for the divergence of T1 morphologies have yet to be elucidated.

Classical studies in *Drosophila*, combined with insights from other holometabolous species such as *Tribolium* and *Bombyx*, have demonstrated that the homeotic gene *Sex combs reduced* (*Scr*) plays key roles in regulating the identity of the T1 and labial segments at both embryonic and adult stages (Abzhanov et al., 2001; Beeman et al., 1989; Curtis et al., 2001; Kokubo et al., 1997; Lewis et al., 1980; Mahaffey and Kaufman, 1987; Pattatucci et al., 1991; Reuter, 1990; Struhl, 1982; Wakimoto and Kaufman, 1981). The primary role of *Scr* is to suppress wing formation on the adult prothorax, a presumed

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ancestral role in insects (Carroll et al., 1995; Rogers et al., 1997; Tomoyasu et al., 2005). While the roles of *Scr* in labial development and comb formation in the fore legs are conserved in *Oncopeltus*, the morphology or identity of the T1 sclerites is unaffected in embryos (Hughes and Kaufman, 2000; Rogers et al., 1997). This observed difference indicates that *Scr* function has been changing over the course of insect evolution and highlights the importance of characterizing its adult function in species that undergo hemimetabolous development.

Hemimetabolous insect species undergo a mode of development in which embryos hatch into first nymphs that resemble a miniature adult. Insights from functional studies, primarily in hemipterans and orthopterans, show that gap and hox genes establish the nymphal body plan during embryogenesis (Angelini and Kaufman, 2004, 2005b; Mahfooz et al., 2007; Rogers et al., 1997). While segment identity and their overall features remain constant, the elaboration of individual segment morphology occurs mainly during post-embryonic development. However, at present, very little is known about the mechanisms that govern segment identity and diversity in adult hemimetabolous insects. This is in contrast to the situation in holometabolous species where it has been shown that input from *Scr* is required throughout development (Beeman et al., 1993; Pattatucci and Kaufman, 1991). The caveat in interpreting these results lies in the fact that immature stages in holometabolous insects (larvae) are generally phenotypically different from adults. The differences between these two modes of development raise two intriguing questions. First, is the identity of segments in hemimetabolous species, once established in first nymphs, irreversible? Second, do hox genes play a role in generating morphological diversity of adults, similar to their recently discovered embryonic function (Mahfooz et al., 2007)?

To begin to address these questions, we examined the post-embryonic functions of *Scr* in the hemimetabolous insect, *Oncopeltus fasciatus* (milkweed bug). In this report we examined the effect of *Scr* depletion during the last two nymphal stages (fourth and fifth) in *Oncopeltus.* Our results show that *Scr* has a primary role in T1 and highlight the importance of the temporal requirements of *Scr* during postembryonic development. As evidenced by the appearance of ectopic wings in individuals that underwent consecutive RNAi treatment (injections at both 4th and 5th nymphs), the abolition of *Scr* at the final two post-embryonic stages is sufficient and necessary to restart the wing program on T1. This is further supported by the fact that ectopic wings were never observed in individuals that were injected at single stages (4th or 5th nymphs). RNAi experiments have also determined that *Scr* is critical for proper formation of T1-specific morphology, especially in the dorsal prothorax. More specifically, the pronotum in *Scr*-RNAi adults displays a range of phenotypes, from significant alterations in its size and shape to a near complete transformation of the posterior half toward a T2-like identity. The latter observation indicates that previously established segmental identities of first nymphs can be altered, and therefore, are not irreversible. At the same time, our analysis also shows that other features that are fully developed at the first nymphal stage (labial tube, leg combs) are unaltered in *Scr*-RNAi adults suggesting that their identities cannot be changed during post-embryogenesis. These results provide a better understanding of what role(s) *Scr* may have played in the development and evolution of the prothorax in adult hemimetabolous insects.

Materials and methods

O. fasciatus rearing

O. fasciatus were reared at room temperature and were provided water on moist towels and fed cracked sunflower seeds. Adult females laid eggs on cotton rolls from which they were collected daily. Embryonic development was complete in approximately 7–8 days at room temperature. Upon hatching, first instars were transferred to new cages and reared on an identical diet as provided for the adults. Each successive molt occurs in approximately 6–7 days until post-embryogenesis is complete. On average, it took about 6–7 weeks for *Oncopeltus* first nymphs to fully develop into adults.

Preparation of Scr dsRNA

The original report of *Scr* expression in *Oncopeltus* (Rogers et al., 1997) utilized a fragment of *Scr* that included the highly conserved homeodomain region. Subsequent analyses of *Scr* function in *Oncopeltus* (Hughes and Kaufman, 2000) utilized a shorter, non-overlapping 3′ fragment of *Scr* that produced specific *Scr*-RNAi phenotypes. This fragment was generously provided by C. Hughes and was used in the present analysis to generate *Scr* dsRNA as described in Mahfooz et al. (2007).

RNA-interference (RNAi)

Adapted from the maternal RNAi protocol by Liu and Kaufman (2004), *Scr* double-stranded RNA (dsRNA) was prepared and injected into the abdomen of adult milkweed bug females using a Hamilton syringe with a 32-gauge needle. Individual females were placed in separate containers with a single male. Eggs were laid and clutches were collected on a daily basis and allowed to mature at room temperature. First nymphal instars hatched after eight days upon which their morphologies were analyzed. Typically, *Scr*-RNAi phenotypes began emerging after the third clutch (first two clutches were mainly wild type). Our embryonic RNAi phenotypes were indistinguishable from those reported by Hughes and Kaufman (2000) and indicate that our mRNAi methodology is effectively and specifically abolishing *Scr* function.

Nymphal RNAi was performed by injecting *Scr* dsRNA into the abdomens of *Oncopeltus* nymphs at either the third, fourth, fifth, or consecutively at both fourth and fifth nymphal stages using a Hamilton syringe with a pulled glass capillary needle. Approximately 2 μl of *Scr* dsRNA was injected into each nymph at a concentration of 2–3 μg/μl. The total numbers of injected nymphs were as follows: 38 third nymphs, 67 fourth nymphs, 51 fifth nymphs, and 25 nymphs injected at both fourth and fifth nymphal stage (consecutive). The proportion of injected nymphs that molted into adults were 0% (third nymphs), 19% (fourth nymphs), 61% (fifth nymphs), and 32% (consecutive). In order to examine further the causes of high mortality observed in third and fourth nymphal injections, we performed a complementary study using *Ubx*-depleted *Oncopeltus* first nymphs (Mahfooz et al., 2007). These nymphs resulted from maternal *Ubx*-RNAi injections, and have a distinct phenotype in T3 and A1 segments. Unlike *Scr*-RNAi first nymphs, *Ubx*-depleted individuals have normal mouthparts and were observed feeding and behaving similar to a wild type. Briefly, of the ~300 *Ubx*-RNAi first nymphs examined, 60% molted into third nymphs. Subsequently, less than 10% of these individuals were able to successfully molt into fourth nymphs, while none survived to the fifth nymphal stage. These data suggest that hox genes such as *Ubx* and *Scr* may perform critical functions during third and fourth nymphal stages of post-embryonic development in *Oncopeltus*, as depletion of either of these genes results in lethality.

Cloning of partial GFP fragment

A 710 bp fragment of the jellyfish Green Fluorescent Protein (GFP) was cloned using the plasmid pRSET-emGFP (gift from P. Cunningham) as a template. Specific primers were designed to previously published sequence data and PCR conditions were used to generate partial cDNA fragments of GFP as described in Li and Popadić (2004). Five clones were isolated, sequenced, and subsequently compared to previously published data revealing a 100% sequence identity. dsRNA

synthesis of the GFP fragment was performed as described in Mahfooz et al. (2007).

To test for the possibility of non-specific effects from our post-embryonic *Scr*-RNAi injections, we injected the GFP dsRNA fragment into the abdomens of 30 third, 15 fourth and 15 fifth stage *Oncopeltus* nymphs. Regardless of the stage of injection, all surviving adults were indistinguishable from wild type. These data indicate that the adult phenotypes observed in our post-embryonic RNAi-*Scr* injected individuals can indeed be specifically attributed to a reduced amount of *Scr* transcript.

RT-PCR analysis

Oncopeltus fourth nymphs were injected with 1–2 μl of *Scr* dsRNA at 2 μg/μl and allowed to molt into fifth nymphs. At this stage, the T1 plates from three individuals (excluding the legs) were dissected and total RNA was extracted utilizing Trizol (GibcoBRL/Life Technologies). This RNA was subsequently used as a template to generate cDNA utilizing a poly-T primer (Promega). For comparison, total RNA and cDNA was generated from wild type T1 plates from three individual *Oncopeltus* fifth nymphs in an identical manner. Equal concentrations of cDNA of both wild type and *Scr*-RNAi fifth nymphs were subsequently used as templates in individual PCR reactions to assess the amount of *Scr* transcript that was abolished in injected individuals. *Scr* primers were designed according to published *Scr* sequence data originally reported by Hughes and Kaufman (2000). As a positive control, primers designed to the *Oncopeltus* 18S ribosomal subunit were used in both wild type and *Scr* injected fifth nymphs. The amount of this fragment should be identical in both instances, as injected *Scr* dsRNA should have no effect on the endogenous levels of this transcript. The PCR conditions were as follows: 94 °C for 3 min; 30 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s; one cycle of 72 °C for 7 min.

Results and discussion

Role of Scr during embryonic development

In insects, *Sex combs reduced* (*Scr*) is expressed and functions in two distinct regions of the body, the head and thorax, establishing identity to unique structures found on these segments during embryonic development (Beeman et al., 1989; Hughes and Kaufman, 2000; Pattatucci and Kaufman, 1991; Rogers et al., 1997). Overall, the embryonic expression pattern of this gene is highly conserved, although some notable variations in its function and regulation were observed between species (Angelini et al., 2005; Hughes and Kaufman, 2000, 2002; Rogers et al., 1997). In *Oncopeltus* embryos, *Scr* is expressed throughout the labial appendages (except the distal tip) and in the posterior region of the maxillary segment (Hughes and Kaufman, 2000; Rogers et al., 1997). In the thorax, *Scr* is localized in a dorsal patch on the prothorax (T1) and in a spot on the distal tibia of T1 legs (Hughes and Kaufman, 2000; Rogers et al., 1997). Subsequent functional analyses have shown that *Scr* primarily affects the establishment of the labial segment during embryonic development, with minor effects on the maxillary appendages and T1 legs (Angelini and Kaufman, 2005c; Angelini et al., 2005; Hughes and Kaufman, 2000; Rogers et al., 1997).

In light of the focus of the present study on examining possible *Scr* functions in the T1 segment, we have independently performed *Scr*-RNA interference (RNAi) and examined the morphology of the prothorax in first nymphs. Consistent with previous reports (Angelini and Kaufman, 2005b; Hughes and Kaufman, 2000), in the absence of *Scr* the labial tube is split into a pair of leg-like appendages, complete with claws (Fig. 1A vs. B). In the T1 legs, the combs at the distal tibia are malformed, being larger in size and reduced in number (Figs. 1C, D). Note that while *Scr* does have a role in fore legs, the overall identity of the T1 segment is not altered in *Scr*-RNAi first nymphs (Figs. 1E, F). There are two key features, segment size and bristle pattern that distinguish the dorsal T2 plate (mesonotum) from its T1 counterpart (pronotum). The mesonotum is narrow while the pronotum is twice as wide (Figs. 1E, F). In addition, the distribution of bristles along the dorso-lateral margin differs between the two segments (Chesebro, 2008). Both of these T1 defining features are retained in *Scr*-RNAi first nymphs. Combined, these results demonstrate that the primary embryonic role of *Scr* is to provide identity to the labium and has no effect on the prothorax in first nymphs.

Post-embryonic development in O. fasciatus

Since the focus of our study is to determine the role(s) *Scr* plays in establishing adult morphologies, the interpretation of our results requires a detailed description of post-embryonic development in *O. fasciatus*. There are a total of six stages of post-embryogenesis in *Oncopeltus* (Fig. 2). The first five are nymphal stages and the last is a sexually mature adult with fully developed, functional wings. Although wings are absent on the first nymphs that hatch from the

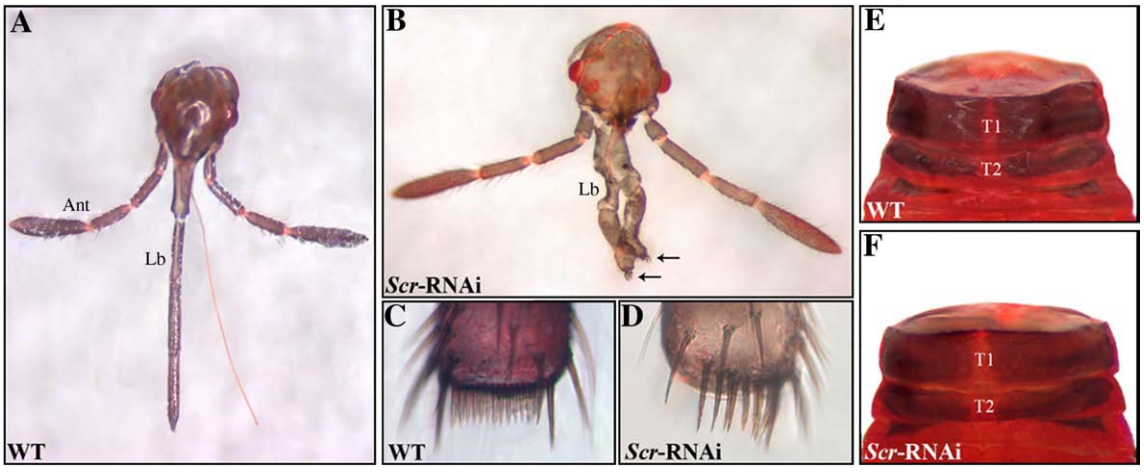


Fig. 1. Embryonic and post-embryonic *Scr*-RNAi phenotypes in *Oncopeltus fasciatus*. (A) Wild type labium of first nymph. The two labial appendages fuse to form one long labial tube. (B) *Scr*-RNAi first nymph. The labial appendages do not fuse and their morphology is altered toward leg-like identity; note the development of claws at the distal tips (black arrows). (C) Wild type T1 leg comb on the distal tibia of first nymph. The 12–14 bristles are short and organized in a neat row. (D) *Scr*-RNAi T1 leg comb. The bristles are longer and fewer in number (6–8) compared to wild type. (E, F) Dorsal views of T1 and T2 segments in a wild type and *Scr*-RNAi first nymphs. Legend: Ant = antenna; Lb = labial appendage; T1 = prothorax; T2 = mesothorax.

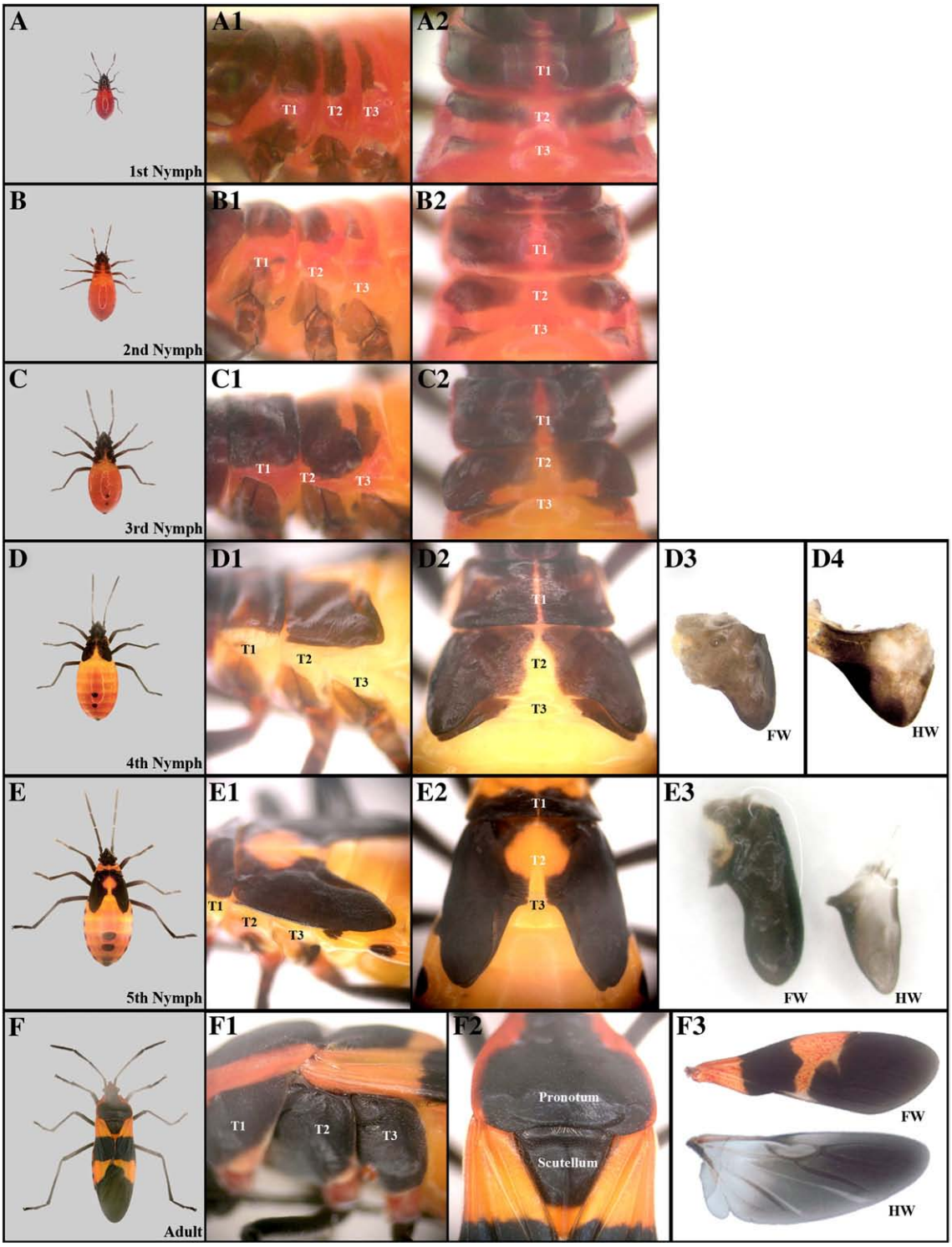


Fig. 2. Wing development in *Oncopeltus fasciatus*. (A–A2, B–B2) Unique stripes of dorsal pigmentation are observed on each thoracic segment at the first (A–A2) and second nymphal stages (B–B2). (C–C2) At the third nymphal stage, the first presumptive winglet is formed on the T2 segment, while the T3 segment retains a narrow stripe of black pigmentation. (D–D4) At the fourth nymphal stage both the fore and hind wings are visible as tiny winglets with distinct shapes. (E–E3) At the fifth nymphal stage both pairs of winglets grow in length and size. By this stage the fore winglets become differentially larger as compared to the hind winglets. (F–F3) At the final stage (adult) the fore and hind wings display differential morphologies in which the fore wings are brightly colored and partially hardened (known as hemelytra) while the hind wings are membranous and uniformly colored. Legend: T1 = prothorax; T2 = mesothorax; T3 = metathorax; FW = fore wing; HW = hind wing.

eggs, unique stripes of dorsal pigmentation are observed on each thoracic segment (Figs. 2A–A2). At the second nymphal stage, the only external change is a moderate increase in body size while the thoracic morphology remains the same (Figs. 2B–B2). However, at the third nymphal stage, the first presumptive winglet is formed on the T2 segment, while the T3 segment retains a narrow stripe of black pigmentation (Figs. 2C–C2). By the fourth and fifth nymphal stages, distinct winglets corresponding to fore and hind wings are clearly noticeable on their respective T2 and T3 segments (Figs. 2D–D2). These winglets can be dissected at the fourth nymphal stage highlighting their different shapes, particularly in their distal regions (Figs. 2D3, D4). By the fifth nymphal stage, the fore and hind winglets

have acquired differences in size, shape, and pigmentation that will be reflected in the adult wings (Figs. 2E–E3).

In contrast to fifth nymphs, the wings in adults exhibit the largest increase in size, with the fore wings completely covering the hind wings and the abdomen (Fig. 2F). In addition to fully functional fore wings, the T2 segment also develops a prominent triangular dorsal plate known as the scutellum that is absent during the nymphal stages (Figs. 2F2, 3G left). Both pairs of wings (fore and hind) have different colors, shapes and venation patterns. The fore wings are brightly colored, with distinct orange and black patterning and are bigger in size than hind wings (Fig. 2F3). Furthermore, the basal (proximal) region of the fore wing is sclerotized (but not as heavily as in coleopterans), while the distal region remains membranous. Such wing structure is known as hemelytra and is a specific characteristic of the order Hemiptera. The hind wings on the other hand, are broader (but smaller) than fore wings and have an elliptical shape at the distal region. They are completely membranous, have a uniform greyish color and are characterized by fewer veins as compared to fore wings (Fig. 2F3). While both pairs of wings are utilized for flying, the fore wings also protect the hind wings when they are folded up at rest.

Post-embryonic function of Scr in Oncopeltus

While it would be advantageous to follow the growth of *Scr*-depleted first nymphs in order to deduce the adult function(s) of *Scr*, this is unfeasible as the nymphs are unable to feed using their abnormal mouthparts (Fig. 1B). Fortunately, wild type *Oncopeltus* first nymphs undergo four subsequent nymphal stages that can be potentially targeted by RNAi. To infer an individual nymphal stage's contribution to adult morphology we injected double stranded *Scr*-RNA at 3rd, 4th, and 5th instars and allowed them to mature into adults. Injected third instars rarely molted into 4th nymphs and never passed successfully into 5th, indicative of a functional requirement for *Scr* at this stage. In a parallel experiment, 30 third instars were independently injected with dsRNA that corresponds to a 710 bp fragment of the jellyfish green fluorescent protein (GFP). This set of injections acts as a negative control since no GFP gene exists in *Oncopeltus*. In contrast to *Scr* third nymphal injections, 53% of third instars that were injected with GFP dsRNA successfully developed into adults and were indistinguishable from wild type. These data show that the 100% lethality observed in *Scr* injected third nymphs is due to the loss of gene function and further supports our finding that the function of this gene at the third nymphal stage is essential for viability in *Oncopeltus*. Injections at either 4th or 5th nymphal stages predominately resulted in moderate adult phenotypes (Fig. 3B), with few exceptions. However, RNAi experiments in which individuals were consecutively injected at both 4th and 5th nymphal stages resulted in a near complete transformation of T1 into T2, suggesting that the effects of post-embryonic *Scr*-RNAi in *Oncopeltus* are cumulative. To determine the effectiveness of our RNAi methodology, injected 4th nymphs were allowed to molt into the next stage upon which their T1 plates were dissected and evaluated for *Scr* mRNA. As shown by RT-PCR analysis in Fig. 3K, only trace levels of *Scr* mRNA are detected in *Scr*-RNAi nymphs compared to wild type. This result confirms that the observed adult phenotypes are, indeed, due to depletion of *Scr*. As depicted in Figs. 3A, B, there are marked differences between embryonic and post-embryonic *Scr*-RNAi phenotypes. The labial segment, which undergoes major change in embryos, is only slightly affected in adults. While the overall morphology of the labial tube remains wild type, the small groove at its base (buccula) fails to form (Figs. 3A1, B1, arrowheads). In a similar fashion, the combs on fore legs are unaffected in *Scr*-RNAi adults (Figs. 3A2, B2). However, the entire prothoracic plate displays major morphological alterations with regard to its size, shape, and pigmentation (Figs. 3B arrowhead; C–F). These results highlight a major change in the primary function of *Scr* between embryonic and post-embryonic development. In the former, the principally affected segment is the labium, with a minor role in forelegs. In the latter, however, the main effect is observed in the prothorax and not in its appendages.

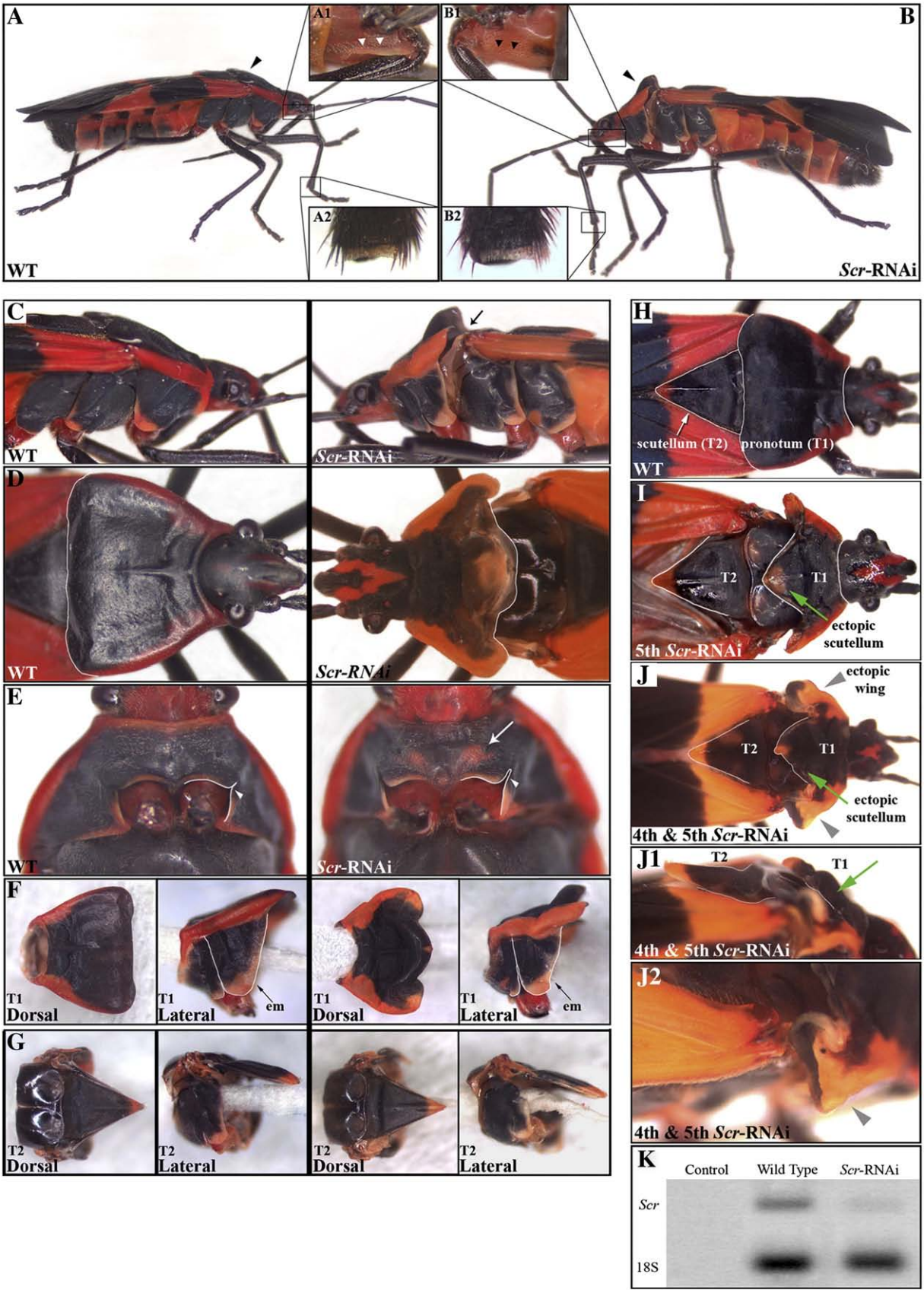
Alterations in the prothorax are the key features of adult Scr-RNAi phenotypes

Three groups of sclerites (plates) compose each thoracic segment, which in the prothorax are the pronotum (dorsal), propleura (two lateral), and the prosternum (ventral). In wild type, the pronotum (Figs. 3C, D, left) is large and smooth, laying flat over the anterior half of the dorsal mesothoracic plate (T2). However, the pronotum of moderate *Scr*-RNAi adults (primarily resulting from injections at either the 4th or 5th nymphal stages) is not flush with the T2 plate and instead becomes wavy, curved on the sides, and elevated in the midsection, exposing the underlying tissue (Figs. 3C, D, right). Additionally, the pigmentation of the pronotum is also modified. The wild type pronotum is solid black with red-orange stripes along the lateral edges. In *Scr*-RNAi adults these lateral stripes are wider dorsally and expand medially at the posterior-most edge (compare dorsal views, Fig. 3F). On the ventral surface, two ectopic red dots appear on the prosternum (Fig. 3E right, white arrow), suggesting a potential role for *Scr* in the regulation of pigmentation on T1. Laterally, the epimeron (posterior propleural plate) is visibly reduced in size and changes shape from triangular to somewhat rectangular (compare lateral views, Fig. 3F). In addition, the separation between the lateral plates increases significantly (white arrowheads, Fig. 3E, left vs. right). Overall, these results indicate that *Scr* controls a whole set of features of prothoracic morphology including shape, size, and pigmentation in adult *Oncopeltus*.

While the majority of individuals injected at either the 4th or 5th nymphal stages were moderately affected, a small percentage of adults displayed strong *Scr*-RNAi phenotypes, characterized by the

Fig. 3. Detailed analysis of *Scr*-RNAi *Oncopeltus* adult phenotypes. (A) Wild type *Oncopeltus* adult. (A1) Wild type adults develop an "oral groove" (buccula) at the base of the labium (white arrowheads). (A2) Wild type adult T1 leg comb. (B) *Scr*-RNAi adult showing an overall change in size and shape of T1 (arrowhead) compared to wild type. (B1) The buccula does not form at the base of the labium in *Scr*-RNAi adults (black arrowheads). (B2) Adult T1 leg combs develop normally in *Scr*-depleted adults. In panels C–G, wild type *Oncopeltus* adults are on the left and *Scr*-RNAi adults are on the right. (C) Lateral view of adult prothorax. In wild type, the T1 plate is smooth and lies flush over the anterior portion of the T2 plate. In the *Scr*-RNAi adult the plate is reduced in size laterally and elevated dorsally, exposing tissue between the T1 and T2 plates (black arrow). (D) Dorsal view of T1 plate. In wild type, the posterior end of the pronotum is smooth and partially overlaps the T2 plate. In the *Scr*-RNAi adult the posterior end of the pronotum is wavy and elevated exposing the anterior mesothorax. (E) Ventral view of the T1 plate. In wild type, the prosternum is solid black in color, where in the *Scr*-RNAi adult the prosternum develops two ectopic red spots (white arrow). Additionally, there is an increase in separation between the two lateral propleural plates (white arrowheads, left and right). (F) Dorsal and lateral views of dissected T1 plates of wild type and *Scr*-RNAi adults. In the dorsal view, the shape of the pronotum changes from round and smooth in wild type (left) to wavy in *Scr*-RNAi adults (right). Additionally, there is a posterior expansion of the red-orange pigmentation along the lateral margins in *Scr*-RNAi adults, as well as the appearance of a red-orange spot at the posterior midpoint. In the lateral view the *Scr*-RNAi adults show a reduction in size of the epimeron and a slight change in its shape (outlined in white). (G) Dorsal and lateral views of dissected T2 plates of wild type and *Scr*-RNAi adults. The mesothoracic segment is unaffected in *Scr*-RNAi individuals and appears as wild type. (H) Dorsal view of wild type adult showing the broad pronotum and the triangular shaped scutellum on posterior T2. (I) Dorsal view of a strong *Scr*-RNAi adult phenotype resulting from a 5th nymph single stage injection. The posterior pronotum is partially transformed toward T2 identity, evidenced by the formation of an ectopic scutellum (green arrow). (J–J2) Phenotype of a consecutively (4th and 5th) injected *Oncopeltus* adult. (J) Dorsal view showing the near complete transformation of T1 toward T2, illustrated by the presence of a well formed ectopic scutellum (green arrow) and ectopic wings on the prothorax (grey arrowheads). (J1) Lateral view of the consecutively inject adult in (J), showing that the ectopic scutellum on T1 (green arrow) is similar in size, shape and pigmentation to that normally found on T2. (J2) Magnified view of the ectopic T1 wing (grey arrowhead) of the consecutive *Scr*-RNAi *Oncopeltus* adult shown in (J). (K) RT-PCR analysis of *Scr* mRNA in fifth nymph prothoracic plates. *Scr*-RNAi nymphs show only trace levels of *Scr* mRNA in T1 compared to wild type. Legend: em = epimeron; T1 = prothorax; T2 = mesothorax.

partial transformation of T1 toward T2. Wild type *Oncopeltus* adults (Fig. 3H) have distinct T1 and T2 morphologies, mainly in the posterior halves of these segments. The pronotum is more box-like and solid black, while the T2 plate tapers into a triangular shape, called the scutellum, that is mostly black with a red-orange tip (Fig. 3H, arrow; G). As depicted in Fig. 3I, strong *Scr*-RNAi phenotypes



resulting from single stage injections (4th or 5th) exhibit a greater reduction of the prothorax as compared to moderately affected individuals (Fig. 3D right). In addition, the posterior half of the pronotum develops an ectopic scutellum of similar size and shape as that on T2, complete with red-orange coloration at the distal tip (Fig. 3I, green arrow). Note, however, that the anterior portion of the pronotum retains its wild type morphology. In contrast, the prothorax of adults that were injected at consecutive nymphal stages (both 4th and 5th) exhibits a near complete transformation of T1 toward T2. These individuals produce a more developed ectopic scutellum on T1 that is not only elevated, but also points toward the posterior in a similar fashion as that on T2 (Fig. 3J1, green arrow). In addition, ectopic wings develop on the prothorax and display an alternating orange and black pigmentation pattern similar to the one that characterizes wild type forewings (Fig. 3J2, grey arrowhead). These results indicate that the suppression of wings on the adult prothorax in *Oncopeltus* requires *Scr* input at both of the last two nymphal stages (4th and 5th). This is further supported by the fact that ectopic wings never develop on the T1 segment of individuals injected a single time at either the 4th or 5th post-embryonic stages. In other words, *Scr* expression at either of the last two nymphal stages is sufficient to suppress the formation of ectopic wings on the prothoracic segment of *Oncopeltus* adults.

Scr and wing repression in Oncopeltus

Previous research in *Drosophila* and *Tribolium* has suggested that *Scr* may have a conserved role in repressing wing formation on T1 in modern insects (Carroll et al., 1995; Rogers et al., 1997; Tomoyasu et al., 2005). The present study in *Oncopeltus* provides the first insight into this putative function in a hemimetabolous species. During post-embryonic development, the winglets that form on the T2 and T3 segments become morphologically distinguishable at the fourth nymphal instar in milkweed bugs (Figs. 2D3, D4). No such structures exist on the prothorax, due to the repression of the wing program during embryonic and the first three post-embryonic stages (nymphs 1–3). Hence, fourth instars are already characterized by fundamental differences in the morphology and function of their thoracic segments. These differences become proportionately larger at the fifth stage, and culminate during the final molt that generates a wingless T1 segment and fore and hind wings on T2–T3 in adults. And yet, as shown by the results of the consecutive *Scr*-RNAi injections (Figs. 3J–J2), the depletion of *Scr* at the last two nymphal stages is sufficient to re-initiate the wing program on T1 despite the complete absence of wing primordia on this segment. Interestingly, the T1 segments of fifth nymphs that emerge from injections at the fourth instar do not exhibit winglets as seen on T2 and T3. Rather, this segment still retains a wild type appearance. Therefore, the ectopic wings that ultimately emerge from the prothorax of consecutively injected individuals do not originate from wing pads but are extensions of the lateral portion of the prothorax (Figs. 3J, J2 grey arrowheads). This result, coupled with the fact that ectopic wings never develop on the prothorax of single stage injections (either 4th or 5th nymphs), suggests that a temporary input of *Scr* at either of the two last post-embryonic stages is sufficient to repress the formation of wings on this segment in *Oncopeltus*. Thus, our data indicates that the ability of *Scr* to suppress wing

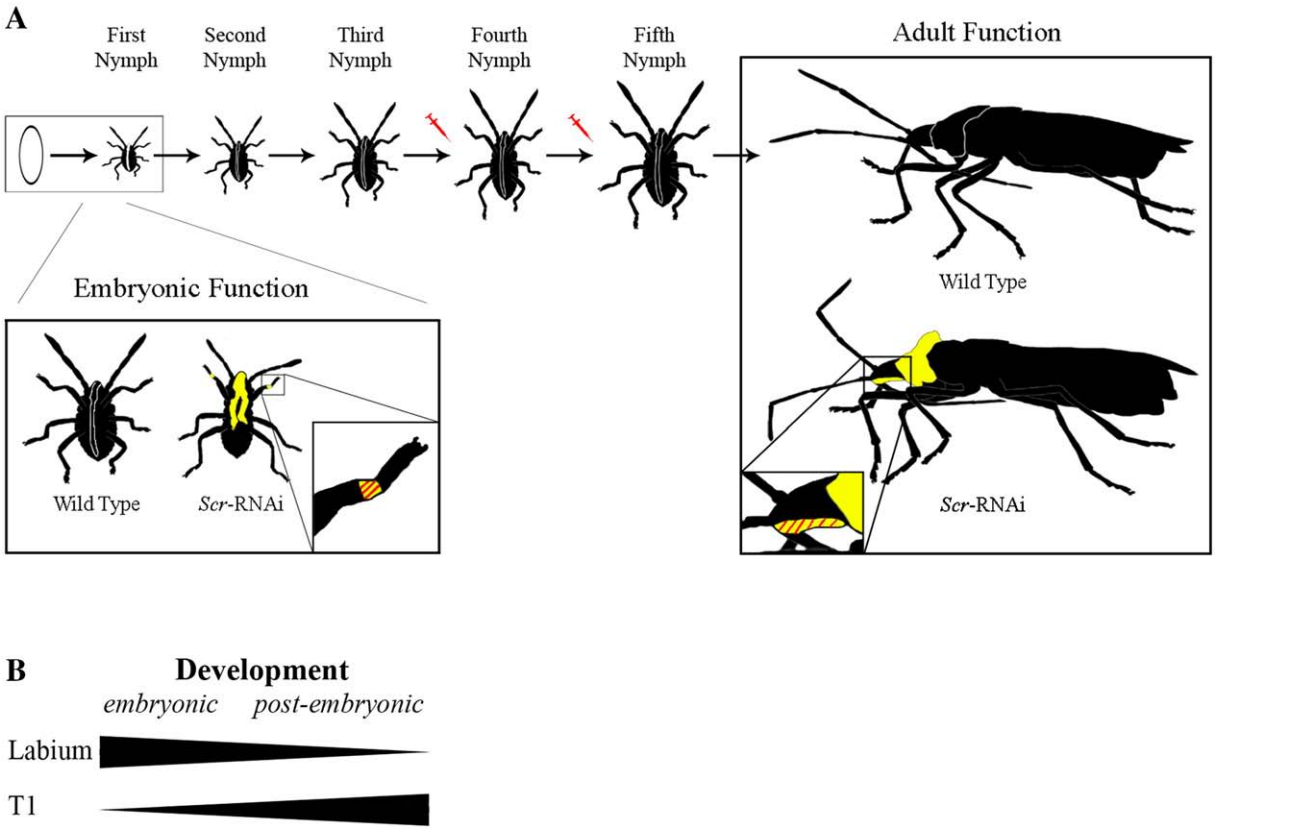


Fig. 4. Summary of the function of *Scr* in establishing embryonic and adult morphologies. (A) During embryogenesis (left; ventral view), the primary role of *Scr* is in providing identity to the labial tube (yellow), while it has a lesser role in the development of the T1 leg combs (red/yellow hatched). In post-embryonic development (right; lateral view), the main function of *Scr* is directing the final morphology of the T1 segment (yellow), with a minor role in the labial "groove" (red/yellow hatched). (B) Graphic diagram representing the general spatiotemporal requirements of *Scr* at various stages of development. In the labial appendages *Scr* is critical during embryonic development, but plays a minor role in this segment post-embryonically. The opposite is true in the prothorax, where the primary function of *Scr* is required post-embryonically, and less so during embryonic development.

development on T1 is conserved and likely represents an ancestral function of this gene.

Divergent functions of Scr during embryonic and post-embryonic development

Currently, the majority of our knowledge into the mechanisms governing the establishment of adult segmental identity is limited to holometabolous insects (Akam, 1987; Beeman et al., 1989; Struhl, 1982; Wakimoto and Kaufman, 1981). This study, therefore, imparts the first insight into the function of a homeotic gene (*Scr*) during post-embryonic development of a hemimetabolous insect species, *O. fasciatus*. As summarized in Fig. 4, our data reveal a divergence in the primary functions of *Scr* between embryonic and post-embryonic development. During embryogenesis (Fig. 4A, left), *Scr* is primarily involved in providing identity to labial appendages and has a more limited role in regulating the formation of combs on T1 legs (Angelini and Kaufman, 2005b; Hughes and Kaufman, 2000, 2002). During post-embryonic development, however, the main function of *Scr* is to regulate the identity of the prothorax with only a minor role in the labial segment (Fig. 4A, right). These results illustrate a major spatial change in the functions of *Scr* between embryonic (main role in labial appendages) and post-embryonic development (main role in the prothorax).

Classic studies in holometabolous species have shown that *Scr* played one of the key roles in the establishment of the subdivision of the insect body into three tagma by regulating the distinct morphology of the T1 segment (Beeman et al., 1989, 1993; Carroll, 1995; Kokubo et al., 1997; Lewis et al., 1980; Mahaffey and Kaufman, 1987; Pattatucci and Kaufman, 1991; Pattatucci et al., 1991; Reuter, 1990; Wakimoto and Kaufman, 1981). Functional analysis of an *Scr* ortholog (*Cephalothorax*) in *Tribolium* resulted in the transformation of the prothorax and T1 legs toward T2 (Beeman et al., 1989). In addition, ectopic wings appear on T1 in both the pupae and adults (Beeman et al., 1989, 1993; Tomoyasu et al., 2005). These results highlight three key aspects of presumed *Scr* functions in insects: first, in regulating the final morphology of the prothorax; second, in wing repression; and third, in controlling the morphology of the forelegs. RNAi experiments in *Oncopeltus* show that these three *Scr* functions are established at distinct developmental stages in *Oncopeltus* (this study, Angelini and Kaufman, 2005b; Hughes and Kaufman, 2000). For example, the final morphology of prothorax is mainly regulated during post-embryonic development. This is best illustrated by the fact that the T1 segments of first nymphs resulting from embryonic *Scr*-RNAi experiments are indistinguishable from wild type, while overt phenotypic changes can be readily observed when *Scr* is depleted at either 4th, 5th or consecutive (4th and 5th) post-embryonic stages. In reference to wing suppression, it is important to note that our *Scr*-RNAi injections were limited to later nymphal stages and only an indirect inference can be made on the importance of *Scr* during early post-embryonic development (1st–3rd nymphal stages). Nonetheless, the fact that we were able to observe the formation of ectopic wings on the T1 segment of consecutively injected nymphs indicates that the depletion of *Scr* at later stages is sufficient to at least partially reactivate the wing program. In addition, the prothorax of consecutively injected individuals exhibit a more complete transformation toward T2 as compared to single stage injections, suggesting that a temporary input of *Scr* at either of the last two post-embryonic stages is sufficient to fully suppress the development of wings on the T1 segment in *Oncopeltus*. As depicted in Figs. 1C, D, the formation of combs on fore legs is affected only by embryonic *Scr*-RNAi injections (Hughes and Kaufman, 2000), while the injections at post-embryonic stages have no effect on this structure (Figs. 3A2, B2). Overall, these results reveal that, in hemimetabolous insects, the three main functions of *Scr* may be temporally separated and restricted to specific stages of development.

Another intriguing aspect of hox gene regulation in hemimetabolous species is the degree to which their input is required during post-embryonic development. That is, if the basic adult body plan and segmental identities are already present at the first nymphal instar, is continuous hox gene input still required during subsequent nymphal stages? Results from *Oncopeltus* indicate that rather than addressing this question in general terms, it may be more appropriate to focus on each segment and its associated structures separately. As an example, the morphology of the labial appendages is essentially the same between first nymphs and adults (except for an increase in size). As shown by *Scr*-RNAi experiments (Angelini and Kaufman, 2005b; Angelini et al., 2005; Hughes and Kaufman, 2000), the identity of the labial appendages is mainly established during embryogenesis,

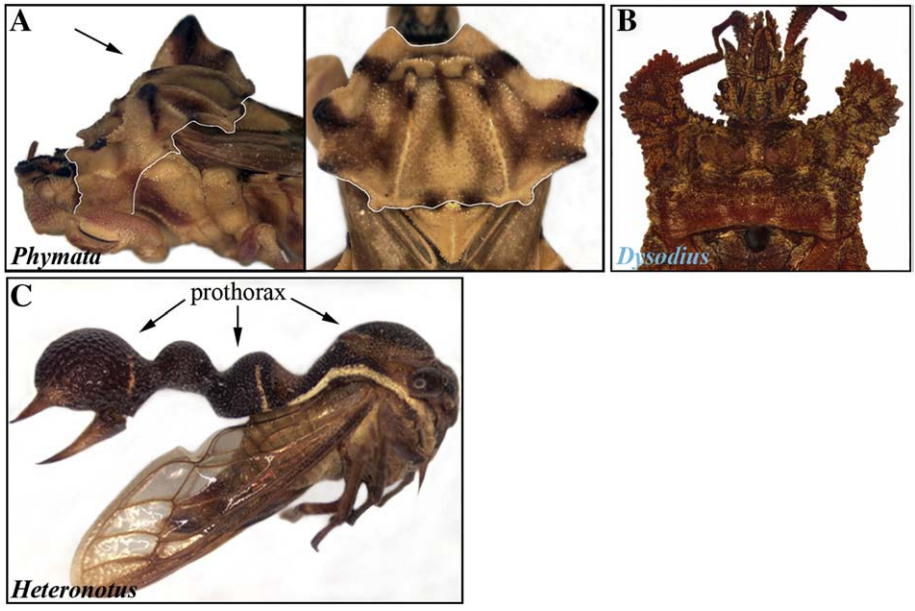


Fig. 5. Representative hemipterans displaying a wide range of prothoracic phenotypes. (A) The ambush bug, *Phymata praestans* (Reduviidae), has a wide pronotum that is slightly wavy and elevated above the body. (B) The pronotum of the flat bug, *Dysodius lunatis* (Aradidae), is serrated with two large outgrowths protruding on either side of the head. (C) The treehopper *Heteronotus trinodosus* (Membracidae) has a greatly enlarged dorsal T1 segment that extends well past the abdomen.

whereas only a minor trait (buccula) is regulated post-embryonically (this study). In a similar manner, the combs on T1 legs are already formed in first nymphs and are morphologically similar to those found on adults. In this instance as well, *Scr* controls comb morphology during embryogenesis and has no function on this structure post-embryonically. In contrast, a very different situation exists with regard to the overall morphology of the T1 plates. In this example, while the identity of T1 is distinct from T2, the key features of this segment are not established during embryogenesis. Instead, the shape, size, and pigmentation of the prothorax are continuously modulated throughout post-embryonic development (Fig. 2). Consistent with this observation, consecutive RNAi-*Scr* injections at both the 4th and 5th nymphal stages resulted in a more complete transformation of T1 toward T2 as compared to single stage injections (4th or 5th). These data suggest that the input of *Scr* at late post-embryonic stages (4th, 5th, consecutive 4th and 5th) is critical for the establishment of adult T1 identity. Overall, these results illustrate that *Scr* function is crucial at stages when the final features of a structure are formed (summarized in Fig. 4B). For example, in the labial appendages and T1 combs, *Scr* is required during embryogenesis while its role diminishes thereafter (subsequent nymphal instars). In the case of the T1 sclerites, *Scr* has a minor role during embryogenesis, but is essential during the last nymphal instars. Hence, in hemimetabolous insects, the post-embryonic input of *Scr* may be required for traits whose final morphologies have not yet been established at the first nymphal stage.

Evolutionary implications

The present study shows that *Scr* governs the formation of distinguishing features on the adult prothorax during post-embryogenesis in *Oncopeltus*. As illustrated in Figs. 3I, J, the strong *Scr*-RNAi phenotype is characterized by the transformation of T1 toward a T2-like identity, consistent with previous observations in *Drosophila* and *Tribolium* (Curtis et al., 2001; Pattatucci et al., 1991; Riley et al., 1987; Tomoyasu et al., 2005). This result supports the concept that the default state for thoracic segments is that of the mesothorax (T2) and that *Scr* input is required for establishing T1-specific identity (Struhl, 1982). At the same time, it is tempting to speculate on the relationship between moderate *Scr*-RNAi phenotypes in *Oncopeltus* (Figs. 3C, D right) and the diversity found in the T1 segment in insects in general. The morphologies of the prothorax in hemipterans vary from shortened and elevated (ambush bug, Fig. 5A) to flattened and serrated (flat bug, Fig. 5B). The most extravagant T1 modifications, by far, can be found in the treehoppers in which an enormous pronotum extends the entire length of the insect's body or even beyond the abdomen (Fig. 5C). The current work in *Oncopeltus* shows that the T1 segment of moderate *Scr*-RNAi milkweed bugs develops a wavy texture reminiscent of the prothorax of the ambush bug (Fig. 5A). This resemblance suggests a possibility that a common *Scr*-triggered mechanism may account for some of the diversity depicted in Fig. 5. Focusing future RNAi studies to species that feature a distinct prothorax will be necessary to elucidate further the putative role of *Scr* in the divergence of adult T1 morphologies.

The advent of wings was perhaps the most significant morphological innovation during insect evolution. While it is commonly considered that wings evolved only once (i.e. are monophyletic), how when and why these structures appeared in insects is a perplexing question that has intrigued biologists for decades (Grimaldi and Engel, 2005). Currently there are two main theories regarding wing evolution: (i) the paranotal lobe theory, and (ii) the exite or gill theory (Grimaldi and Engel, 2005). The paranotal theory suggests that wings evolved from extensions of the thoracic terga called paranotal lobes (Grimaldi and Engel, 2005; Hamilton, 1971; Quartau, 1986; Snodgrass, 1935). In contrast, the exite or gill theory proposes that insect wings evolved by the modification of pre-existing limb branches of ancestral appendages that probably were first modified into gills, and then eventually into wings (Averof and Cohen, 1997; Grimaldi and Engel, 2005; Kukalova-Peck, 1991; Wigglesworth, 1973). The latter theory has been supported by molecular data showing that two genes that have wing-specific functions in insects are also expressed in dorsally located limb branches (epipodites) that have respiratory and osmoregulatory functions in two crustaceans (Averof and Cohen, 1997). However, the homology of divergent structures can never be proven with absolute certainty (Averof and Cohen, 1997) and, therefore, an insect model system is necessary to truly delineate the evolutionary origin of wings.

As this study shows, hemimetabolous insects offer an opportunity to genetically manipulate wing development during post-embryogenesis. In particular, the normally wingless prothoracic segment can provide an insight into how wings can develop *de nuovo*. Hence, it is now possible to compare and contrast the development of normal wing primordia on the T2 and T3 segments with those that appear ectopically on T1. Utilizing these ectopic structures to study wing initiation on a cellular and genetic level will be key to testing the paranotal theory. Specifically, comparative gene expression and cellular differentiation patterns between T2 and ectopic T1 wings can determine whether the normal processes are recapitulated in the ectopic structure, and hence, test the hypothesis that wings may be derived from thoracic plates.

Acknowledgments

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